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**Nutritional stress induces exchange of cell material and energetic coupling between bacterial species.**

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Knowledge of the behavior of bacterial communities is crucial for understanding biogeochemical cycles and developing environmental biotechnology. Here we demonstrate the existence of a consortium between two anaerobic bacteria, *Clostridium acetobutylicum* (Gram-positive) and *Desulfovibrio vulgaris* Hildenborough (Gram-negative, sulfate-reducing) in which physical interactions between the two partners induce emergent properties. Molecular and cellular approaches show that tight cell-cell interactions are associated with an exchange of molecules, including proteins, which allows the growth of one partner (*D. vulgaris*) in spite of the shortage of nutrients. This physical interaction induces changes in expression of two genes encoding enzymes at the pyruvate crossroads, with concomitant changes in the distribution of metabolic fluxes, and allows a substantial increase in H<sub>2</sub> production without requiring genetic engineering. The stress induced by the shortage of nutrients of *D. vulgaris* appears to trigger the interaction.

Bacteria have usually been studied in single culture in rich media or in specific starvation conditions. These studies have contributed to understanding and characterizing their metabolism. However, they coexist in nature with other microorganisms, and form consortia in which they interact to build an advanced society<sup>1</sup> that drives key biogeochemical cycles<sup>2</sup>. These consortia vary in space and time as a function of the nutrients<sup>3-4</sup>. In such microbial networks, microorganisms can develop different types of interaction, such as exchange of metabolites. The observed phenotype results from these interactions and not from the individual genotypes alone<sup>5</sup>. The interaction with other species allows microorganisms to occupy environmental niches that would otherwise be closed to them and, depending on the ecological niche occupied; they can show different properties and functions. Mixed cultures have accordingly broader metabolic capabilities and greater robustness to environmental fluctuations than single cultures, characteristics that are attractive for biotechnology<sup>6-7</sup>. However, our knowledge of the physical

and metabolic interactions that help microbes to survive and to develop in a complex network is limited.

In anaerobic environments microorganisms cooperate in the feeding chains, and the complete degradation of complex matter to methane and carbon dioxide involves at least four groups of microorganisms, including primary and secondary fermenters and at least two types of methanogenic archaea<sup>8</sup>. Many studies have examined the syntrophic growth of sulfate-reducing bacteria (SRB) acting like secondary fermenters and methanogenic archaea<sup>9-11</sup>. Most recently, the metabolic interaction between Clostridia, acting as primary fermenters, and methanogenic archaea has also been studied<sup>12-13</sup>, highlighting a symbiotic process of interspecies hydrogen transfer that does not require physical interaction. However, very few studies have examined interaction between SRB and Clostridia, and little is known about the complex interactions that occur in natural bacterial consortia and how primary fermenter bacteria such as Clostridia compete with other anaerobes like SRB<sup>8</sup>.

We have accordingly constructed an artificial consortium constituted by *Desulfovibrio vulgaris* Hildenborough (*D. vulgaris*) and *Clostridium acetobutylicum* (*C. acetobutylicum*). These bacteria are representative of species found in the same environment and are both involved in biomass degradation<sup>8</sup>. *D. vulgaris* Hildenborough is a Gram-negative SRB which uses sulfate as a terminal electron acceptor for the heterotrophic oxidation of organic compounds or hydrogen to produce sulfide<sup>14</sup>. *C. acetobutylicum* is a strictly fermentative endospore-forming Gram-positive bacterium. It converts sugars and starch to organic acids (acetate, butyrate and lactate) during acidogenesis, and solvents (acetone and butanol) during solventogenesis<sup>15-17</sup>. Here we show that the condition of nutriment starvation for *D. vulgaris* in the co-culture induces interspecies cell-cell interaction, allowing exchange of cytoplasmic material (tested with fluorescent molecules, including proteins), associated with metabolic changes and much higher production of H<sub>2</sub>. The metabolic changes suggest that the understanding of microbial consortia



may offer a type of ecological engineering of microbial ecosystems that could provide new approaches for modifying or controlling metabolic pathways.

## **Results**

**Physical contact is needed for growth of *D. vulgaris*** We defined a medium (GY) that allowed growth of *C. acetobutylicum* and survival of *D. vulgaris*. Both strains have been grown on their classical media (Starkey medium for *D. vulgaris* and 2YTG medium for *C. acetobutylicum*), and then washed three times before inoculation. Strikingly, when the two strains were present together, maximum growth (estimated by optical density) occurred earlier than seen for growth of *C. acetobutylicum* in a pure culture (Fig. 1a). The growth pattern follows glucose consumption (Fig. 1b). As expected, there was no growth of *D. vulgaris* in single culture, in GY medium, as *D. vulgaris* cannot grow on glucose or other hexoses, because of lack of permeases<sup>18</sup>. Survival of *D. vulgaris* in single culture in GY medium can be explained by its capacity to accumulate and to use polyglucose and elemental sulfur particles when necessary<sup>19</sup>. Its capacity to use sulfur particles was confirmed by the production of H<sub>2</sub>S at a concentration of 0.15 mM (at the end of the exponential phase), higher than that of 0.035 mM sulfate derived from the inorganic nutrients and yeast extract present in the GY medium. The coexistence of the two bacteria in the co-culture in GY medium was assessed using specific gene markers and cell-viability experiments (Supplementary Figure 1). The growth of *C. acetobutylicum* and *D. vulgaris* in the co-culture was confirmed by flow cytometry, taking advantage of their difference in shape (Supplementary Figure 2). In addition, dynamics of the two strains in the co-culture were monitored with q-PCR. Figs 1c and d highlight the fact that *D. vulgaris* grows only in the presence of *C. acetobutylicum*, as the number of genome copies increases with time. As expected, *C. acetobutylicum* grows both in single culture and in co-culture. The progressive formation of cell aggregates may explain why in supplementary Fig. 2a there is only one peak at 30 h.

To better understand the growth of *D. vulgaris* in the co-culture, we tested whether *D. vulgaris* could use the metabolites produced by *C. acetobutylicum*, and if their presence would allow *D. vulgaris* growth. *C. acetobutylicum* was grown in the GY medium and at various times cells were removed and a washed suspension of *D. vulgaris* was inoculated. No *D. vulgaris* growth was detected after 30 h, at any ratio of glucose to metabolite products, suggesting that *D. vulgaris* cannot use the metabolites produced, even when it was inoculated after 36 h. of *C. acetobutylicum* growth, when the butyrate concentration was 7.7 mM, the acetate concentration 4.6 mM and the lactate concentration 2.3 mM (and no glucose). Furthermore, the addition of 20 mM lactate to the GY medium does not allow the growth of *D. vulgaris* in pure culture (Supplementary Table 1). The presence of *C. acetobutylicum* cells appears necessary for the growth of *D. vulgaris*. It could be argued, however, that in these experiments the H<sub>2</sub> and the CO<sub>2</sub> produced by *C. acetobutylicum* had been lost. The two bacteria were accordingly co-cultivated with either *D. vulgaris* or *C. acetobutylicum* confined in a dialysis tube (Figs 1h, 1i) which prevents physical interspecies interaction, but not the diffusion of small molecules, such as the metabolites, or small proteins (< 7kDa). Whether *D. vulgaris* was in the dialysis tube or outside, there was no growth (Figs 1e, 1g); the culture behaved like pure *D. vulgaris* culture. When *C. acetobutylicum* was in the outside medium and *D. vulgaris* in the dialysis bag (Fig. 1i), the growth profile of *C. acetobutylicum* was not significantly altered in relation to the pure culture (Figs 1e, 1g); there was no modification in glucose consumption either (Fig. 1f). These experiments suggest that the growth of *D. vulgaris* requires the two bacteria to be present in the same compartment and most probably in conditions that allow cell-cell interaction, as the element which allows *D. vulgaris* growth appears not to be freely diffusible through a dialysis membrane.

In the absence of yeast extract in the culture medium, there was no growth with any of the pure cultures. The co-culture however presented a small but significant growth (Supplementary Table 1). This suggests, as proposed by Kato<sup>20</sup> in the case of syntrophic co-culture of *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*, that amino acids and coenzymes can be transferred between *C. acetobutylicum* and *D. vulgaris*.

**Exchange of cytoplasmic molecules between the bacteria** Networks of bacterial cells were observed in the co-culture (Supplementary Figure 3) and the occurrence of physical contact between the two strains was confirmed by flow cytometry (Supplementary Figure 2). A possible non-specific aggregation of the two bacteria, a well known phenomenon<sup>21</sup>, can be ruled out as no co-aggregation is observed in the pure cultures as the peaks are not displaced (Supplementary Figure 2) or when the two pure cultures are mixed, unlike for *Myxococcus xanthus*<sup>22</sup> when aggregate appeared.

The physical interaction between *D. vulgaris* and *C. acetobutylicum* in the co-culture was further investigated by fluorescence microscopy. As *D. vulgaris* is Gram-negative and *C. acetobutylicum* Gram-positive with an external wall of peptidoglycan, the co-culture was labeled with fluorochromes to specifically mark the cytoplasmic membrane (red fluorescence) and peptidoglycan wall (green fluorescence) (Supplementary Figure 4). Fluorescence microscopy revealed zones where the two bacteria interacted, these being apparently associated with a decrease in the green fluorescence (marker of peptidoglycan), suggesting either a partial loss of peptidoglycan after the interaction or an interaction where peptidoglycan was missing (Supplementary Figure 4b). However, the possibility that the decrease in fluorescence is due to differences in the focal planes of the cells cannot be ruled out. This interaction was further analyzed by SEM (Fig. 2). Conglomerates of the two types of bacteria were again observed in the co-culture (Fig. 2c) and in some cases, cell-cell interactions (Fig. 2d) are reminiscent of those described by Dubey<sup>23</sup>

The tight cell-cell interactions suggested the possibility of intercellular exchange of molecules. This was investigated using calcein-labeled *D. vulgaris* or *C. acetobutylicum*, as described in Material and Methods. When *D. vulgaris* was incubated in Starkey medium with calcein-AM a strong green fluorescence was observed after 2h incubation at 37°C indicating calcein sequestration in the cytoplasm. Fluorescent *D. vulgaris* was washed, mixed with unlabeled *C. acetobutylicum* and the mixture was directly visualized by fluorescence microscopy or diluted in fresh GY medium. At time zero, only *D. vulgaris* was fluorescent (Fig. 3a), but after 24h a fluorescence signal was observed from *C. acetobutylicum* in interaction with *D. vulgaris* (Fig. 3b and Supplementary Figure 5a which illustrates a bigger field). However, *C. acetobutylicum* cells not in contact with *D. vulgaris* display little or no fluorescence (Fig. 3c). The response was displayed by 80 to 90% of *C. acetobutylicum* cells (percentages estimated from multiple random fields in three separate biological replicates). There was a similar transfer of fluorescence with calcein-labeled *C. acetobutylicum* mixed with unlabeled *D. vulgaris* (Figs 3d, 3e and Supplementary Figure 5b). Thus calcein can be transferred between *C. acetobutylicum* cytoplasm and *D. vulgaris* in either direction.

To test whether the two bacteria could exchange larger molecules than calcein, such as proteins, we examined whether *D. vulgaris* could exchange mCherry molecules (or GFP) with *C. acetobutylicum*. For that purpose, *D. vulgaris* harboring a pRD3 plasmid containing the gene *mCherry* that codes for mCherry was mixed with *C. acetobutylicum* cells lacking the *mcherry* gene but labeled with calcein, and the co-culture was analyzed by microscopy. At time zero *C. acetobutylicum* shows a green fluorescence and *D. vulgaris* a red fluorescence (Fig. 4a), but after 24 hours, both bacteria show both green and red fluorescence, indicating the presence of mCherry in *C. acetobutylicum* and calcein in *D. vulgaris* (Fig. 4a). Figure 4b illustrates results from another equivalent experiment, and the white arrows point to the connections between *C.*

*acetobutylicum* and *D. vulgaris*; these connecting structures in figs 4a and 4b are consistent with the SEM images.

A similar experiment, but using unlabeled *C. acetobutylicum*, was done with *D. vulgaris* harboring a pRD2 plasmid containing the gene *gfp* that codes for GFP. At time zero only *D. vulgaris* shows green fluorescence, but after 24h, a green fluorescence from *C. acetobutylicum* was also observed, indicating the presence of GFP in *C. acetobutylicum* and in *D. vulgaris* (Supplementary Figure 6)

The possibility that the red fluorescence associated with *C. acetobutylicum* would be due to acquisition of the pRD3 plasmid from *D. vulgaris* through conjugation, can be ruled out, as *C. acetobutylicum* strain ATCC 824 contains a Cac824I restriction endonuclease, which prevents transformation of *C. acetobutylicum* with shuttle vectors used in other bacteria<sup>24-25</sup>. The pRD3 and pRD2 plasmids used in our study contain many Cac824I sites<sup>26</sup> and in consequence they are unstable in *C. acetobutylicum*. This supports the view that the fluorescence associated with *C. acetobutylicum* cells is due to the transfer of mCherry or GFP from *D. vulgaris* to *C. acetobutylicum*.

When the experiments with calcein-AM were repeated with either *D. vulgaris* or *C. acetobutylicum* confined in the dialysis tube to prevent physical interspecies interaction (as in Figs 1h and 1g), there was no fluorescence transfer after 24h (Figs 5a, 5b) indicating that the fluorescent molecules cannot enter or leave the cells *via* the medium culture.

No nutrient known to be used by *D. vulgaris* was present in the GY culture medium in any of the exchange experiments (Figs 3, 4). Addition of lactate (20 mM) alone, a fermentative substrate for *D. vulgaris*, did not modify significantly the behavior of the co-culture (Supplementary Table 1) and calcein fluorescence transfer from *C. acetobutylicum* to *D. vulgaris* was still observed after 24h of co-culture (Fig. 5c). However addition to the culture medium of lactate (20 mM), used as electron donor and carbon source and sulfate (20 mM, electron acceptor) which allows sulfur

respiration metabolism in *D. vulgaris*, prevents or at least greatly reduced the interspecies molecular exchange, as there appears to be no transfer of fluorescence in this case (Fig. 5d). No tight contact similar to that observed without lactate and sulfate (Fig. 2) was detected by SEM (Fig. 5e). These results support the hypothesis that the cell-cell interactions and material transfer are a response to the starvation conditions of *D. vulgaris*. The fact that lactate alone does not inhibit the transfer means that *D. vulgaris* cannot use this nutriment properly, as could be expected in view of the high H<sub>2</sub> concentration present, and in consequence the starvation conditions persist. Control experiments show that lactate, sulfate, or both, have no effect on single culture of *C. acetobutylicum* (Supplementary Table 1).

The hypothesis that the shortage of nutrients can be the triggering factor for the cytoplasmic exchange was explored further through experiments with *E. coli* labeled with mCherry, and co-cultivated with either *C. acetobutylicum* (Fig. 6) or *D. vulgaris* (Fig. 7) labeled with calcein. In co-culture of *E. coli* plus *C. acetobutylicum*, both bacteria grew, but in contrast to what was observed with *D. vulgaris* and *C. acetobutylicum*, no interaction and no exchange of fluorescent molecules was observed (Fig. 6). In this case there is no nutritional stress for either of the bacteria as *E. coli* can use glucose for its growth. This suggests that bacterial interactions in synthetic microbial consortia may emerge as a consequence of individual species solving their own problems of allocation of metabolic resources. Furthermore, this conclusion is supported by the co-culture experiment of *E. coli* plus *D. vulgaris*, (Fig. 7) where the two bacteria grew and interact together, with exchange of fluorescent molecules. Notice the great quantity of bacteria that contain both labels (yellow) illustrated in Fig. 7b.

**Modification of hydrogen metabolism in the co-culture** In the co-culture, as well as in the single *C. acetobutylicum* culture, the growth and production of metabolites resulted from the complete consumption of glucose (Figs 1b, 8a). More than 90% of the electron equivalents were recovered (Supplementary Figure 7). This suggests that all the main metabolites have been

detected, and that any others that could have been produced were at concentrations below the detection threshold, and consequently negligible as carbon sources though they could still act as signals. No solvents (ethanol, acetone, butanol) were detected except occasionally in very small amounts. This observation is in agreement with the experimental conditions used, as production of solvents occurs at much higher concentrations of glucose (typically around 500 mM, much more than the 14 mM used in our experiments), when the pH is lower (4.5 instead of 5.5 in our experiments) and when acids are reassimilated in the stationary phase, which occurs only when acid concentrations are at least around 60 mM<sup>27</sup>.

Single culture of *D. vulgaris* did not consume glucose (Fig. 1b) and no metabolites were detected, in agreement with absence of growth. The same organic acids, *i.e.* lactate, acetate and butyrate, were detected and in similar concentrations in the pure *C. acetobutylicum* culture and in the co-culture, except for the lactate concentration, which decreased by half in the co-culture: this could imply more reducing power for H<sub>2</sub> production. In line with this possibility, the concentrations of H<sub>2</sub> and CO<sub>2</sub> in the co-culture were substantially higher (Figs 8b, 8d), with a H<sub>2</sub> yield about three times that of the single culture, reaching 3.46 mol H<sub>2</sub>/mol of glucose consumed. Due to the high concentration of H<sub>2</sub> in the co-culture, the alternative possibility of H<sub>2</sub> production by *D. vulgaris* via lactate (produced by *C. acetobutylicum*) fermentation is not probable<sup>28</sup> and in addition there was no growth of *D. vulgaris* in pure culture in the GY medium supplemented with 20mM lactate (Supplementary Table 1). The fact that the H<sub>2</sub>/CO<sub>2</sub> ratio was rather similar in single culture or co-culture, suggests an unchanged flux distribution between acetate and butyrate pathways in *C. acetobutylicum*. To get insight into the metabolic changes in *C. acetobutylicum* in the co-culture, reverse transcription PCR was applied to specific genes coding for lactate dehydrogenase (*ldh*) and pyruvate ferredoxin oxidoreductase (*pfor*), the enzymes in the branch point of pyruvate (Supplementary Fig. 8). Changes in their relative activities can alter the flux distribution and thus vary H<sub>2</sub> production. Gene expression studies showed that at 30 hours of



growth of the co-culture, expression of the *pfor* gene considerably increased by a factor of  $6.5 \pm 0.5$ , whereas the *ldh* gene was down-regulated by a factor of  $0.3 \pm 0.1$  (comparison between single culture and co-culture). Assuming that the enzyme activities are proportional to the amounts of mRNA this could produce higher flux distribution from pyruvate to acetyl-CoA than to lactate, thereby increasing the amount of reduced ferredoxin necessary for the  $H_2$  production. The substantial up-regulation of the *pfor* gene together with the down-regulation of the *ldh* gene could also explain why a constant level of the lactate concentration is reached before the glucose is totally consumed (Fig. 8c). *D. vulgaris* gene expression studies are not possible in our conditions (using glucose as energy source) as *D. vulgaris* does not grow in pure culture under these conditions and in consequence no strict control reference can be obtained.

Cell-interaction between *D. vulgaris* and *C. acetobutylicum* appears to be an essential requisite for the increase in  $H_2$  production as no significative increase was observed when the two bacteria were separated by a membrane (Fig. 8f) except in some cases a shorter lag which can be explain by a better anaerobiosis.

## **Discussion**

We show here the establishment in batch culture of a bacterial community constituted of *C. acetobutylicum* and *D. vulgaris*, which (i) can grow without a complete set of nutrients essential for growth of *D. vulgaris* in pure culture, (ii) shows interspecies interaction through physical contact, and (iii) allows interspecies exchange of cytoplasmic material. This behavior permits the emergence of a new phenotype in bacterial communities because it induces modifications of gene expression and changes in metabolism: the mixed community has properties that are not simply the sum of the parts. The physical interaction between the two types of bacteria allows the exchange of molecules of different types and sizes, as different as calcein and GFP or mCherry. Analysis of the genomes of the two bacteria allows us to rule out transport *via* a type IV secretion system, even modified, as no gene encoding proteins involved in this secretion type system are



detected in the genomes. Moreover, such transport needs specific sequence motifs that are not present in GFP or mCherry<sup>29-30</sup>.

Transport mechanisms are in general vectorial, but here the interspecies molecular exchange is bidirectional, which could support the idea of connections between cells that allows a free-exchange. This type of connection could be more easily established in zones where the peptidoglycan wall is absent or thinner.

Cell-cell interaction appears to be an essential requisite for growth of *D. vulgaris* and the increase in H<sub>2</sub> production, the latter resulting from changes in metabolic fluxes at the pyruvate crossroads as no growth of *D. vulgaris* or increase in H<sub>2</sub> production was observed when the two bacteria were separated by a membrane. The absence in the culture medium of substrates necessary for respiratory growth of *D. vulgaris* appears to be the factor triggering the physical contact between *D. vulgaris* and *C. acetobutylicum* as the presence of nutriments, *i.e.* lactate and sulfate, prevents the cell-cell interaction. These results agree with the fact that starvation is an important trigger of stress responses<sup>31</sup>, and is often associated with changes in metabolic pathways<sup>32</sup>. It is highly probable that any shortage of nutrients induces a general stress signal in the first stage of growth that here induces interaction. The fact that there is an exchange of molecules between *D. vulgaris* and *E. coli* but not between *C. acetobutylicum* and *E. coli* is compatible with this hypothesis, because as *E. coli*, like *C. acetobutylicum*, is able to ferment glucose, is not suffering a nutrient stress condition in the GY medium.

The communication between bacteria, which can take several forms<sup>33-38</sup>, is essential for coordinating the behavior of the whole community. Thus communication between cells in pure culture regulates the population density by emitting, receiving and responding to a chemical signal, *i.e.* quorum sensing<sup>39-40</sup>. Other studies have demonstrated a transfer of outer-membrane proteins between two strains of *Myxococcus xanthus*, a wild type strain acting as donor cell and a mutant strain acting as recipient for the product of the missing gene<sup>41-42</sup>. A form of bacterial

communication has been described between adjacent cells *via* connecting “nanotubes”, even between Gram-positive and Gram-negative bacteria<sup>23</sup>, which could resemble the cell-cell interaction between *C. acetobutylicum* and *D. vulgaris* described here, where we show a bidirectional exchange. In anaerobic systems, it has long been thought that interspecies electron transfer is due to interspecies transfer of H<sub>2</sub> or formate<sup>43</sup>. This is well described in the syntrophic behavior between SRB and methanogens, where no physical interaction is required because the H<sub>2</sub> produced by the SRB can diffuse freely to be consumed by the methanogens, which act as scavengers<sup>43</sup> although as any diffusion process, vicinity favors the transfer. Here, on the contrary tight interaction is indispensable.

It is striking that *D. vulgaris* can grow without lactate and sulfate, but with glucose instead, when in the presence of another type of bacteria that can metabolize glucose, such as *C. acetobutylicum* (or *E. coli*). Which nutrients does *D. vulgaris* use in the co-culture? Whatever they are, they must come from *C. acetobutylicum* because only *C. acetobutylicum* can use glucose. An attractive possibility is that molecules from *C. acetobutylicum* could act as electron acceptors, replacing sulfate. As we have shown that proteins can be transferred from *D. vulgaris* to *C. acetobutylicum* it may be that electron shuttles such as ferredoxins could act as electron acceptors in *D. vulgaris* and electron donors in *C. acetobutylicum*. This could result in the production of additional H<sub>2</sub>. This hypothesis is compatible with the fact that *D. vulgaris* does not grow when physical interactions are blocked. Several authors have demonstrated that direct electron transfer might also take place through nanowires<sup>44-47</sup> and it seems to be the primary way of electron transfer in some methanogenic environments. However, proteins known to be involved in this process are absent from *D. vulgaris* and *C. acetobutylicum*. The strategy developed by the bacteria in the present case is different and consists in an energetic coupling involving tight physical interactions and transfer of cytoplasmic molecules with *C. acetobutylicum* (or *E. coli*), and this energetic coupling occurs only in absence of *D. vulgaris*

respiratory substrates. It appears that the type of communication established depends on the environmental conditions and the type of partners involved. Here, we demonstrate that another strategy, different from “classical” syntrophy, exists for bacteria and allows them to grow in absence of substrate.

The significant increase in H<sub>2</sub> production in the mixed community arose without any genetic engineering. This is striking, as increases in H<sub>2</sub> yield normally require artificial construction of several mutants that disrupt competing metabolic pathways<sup>48-49</sup>. Interestingly, the variation in gene expression developed by our co-culture is the same strategy that a biotechnologist would follow: inactivation of pathways that drain the pyruvate pool. So, despite the uncertainty about the mechanisms behind the changes in gene expression, the establishment of bacterial communities with interaction similar to that between *C. acetobutylicum* and *D. vulgaris* could provide a route for improving biotechnological strategies, as it allows significant modifications of gene expression without genetic engineering. This sort of ecological or community engineering of microbial ecosystems could be the tool that biotechnology needs to develop.

Furthermore, the close interaction allowing the exchange of cellular molecules, including proteins, between bacteria as different as *D. vulgaris* and *C. acetobutylicum*, or *D. vulgaris* and *E. coli*, which allows the survival of *D. vulgaris* in the absence of its nutrients and the establishment of a consortium, suggest that bacteria can behave as multicellular organisms in conditions of stress that are closer to what happens in nature, where the availability of nutrients varies considerably.

## **Methods**

**Bacterial strains** *D. vulgaris* was from NCIMB 8303 and *C. acetobutylicum* ATCC824 was kindly supplied by C. Tardif (LCB, UMR7283 CNRS-AMU, Marseilles).

**Media and growth conditions** Strains were grown to steady state in Hungate tubes under anaerobic conditions, in Starkey medium<sup>10</sup> for *D. vulgaris*, and 2YTG medium for *C. acetobutylicum*<sup>17</sup>. The growth medium (Glucose-Yeast extract (GY) medium) used for studying

the consortium was prepared with glucose (14 mM), 0.1% yeast extract, and supplemented with the similar inorganic nutrients used for the Starkey preparation ( $\text{MgCl}_2$  instead of  $\text{MgSO}_4$ ). GY medium was inoculated (10 %) with either washed *D. vulgaris* or *C. acetobutylicum* or with the two strains to constitute an artificial consortium in a 1:1 ratio according to the absorbance at 600 nm. In some cases, the medium of growth was supplemented with 20 mM lactate or/and 20 mM sulfate. Some experiments were carried out with a dialysis membrane (molecular mass cut-off of 7 kDa) in order to physically isolate the two partners. One of the two bacteria, *i.e.* *D. vulgaris* or *C. acetobutylicum*, was placed into a dialysis tube and the other outside the dialysis tube. Controls were done with one type of bacterium in the dialysis tube, and only medium outside. To test the viability of the cells after growth in the GY medium, as single cultures or the co-culture, the specific medium of each bacterium was used, and the growth was followed under anaerobic conditions. The sustainability of the co-culture was explored by taking an inoculum after 30 hours of culture in GY medium and incubating it again in GY medium. After two transfers, *D. vulgaris* and *C. acetobutylicum* still coexisted. The experiments were carried out at least in triplicate.  $\text{H}_2\text{S}$  concentration was determined as described in Giuliani *et al*<sup>50</sup>

**Real Time PCR Quantification (q-PCR)** After extraction of genomic DNA from *D. vulgaris* and *C. acetobutylicum* using the Wizard Genomic DNA Purification Kit (Promega), DNA purity and concentration were determined and the number of copies was determined by q-PCR as described by Savichtcheva *et al*<sup>51</sup>. The primers used for the q-PCR (*dsrA* and *endoG*) are listed in Supplementary Table 2. The reaction was performed with a Kit BioRad SsoFast Eva green Super Mix 2x. The Q-PCR was carried out in CFX 96 BioRad as follows: 2 min 30 s at 98°C for initial activation of enzymes, 45 cycles of 5 s at 98°C, 10 s at 58°C, 2 s at 72°C. Experiments were made in triplicate.

**Reverse Transcription PCR (RT-PCR)** RT-PCR was performed according to Fiévet *et al*<sup>52</sup> for estimating the level of expression of genes coding pyruvate ferredoxin oxidoreductase and

lactate dehydrogenase. 10 µg of total extracted RNA was reverse-transcribed with random hexamers. Real-time PCR was done on a LightCyclerFastStart DNA MasterPlus SYBR green I Kit (Roche Diagnostics). cDNA was mixed with 0.25 µM of each primer and 2 µl of “master mix” in a final volume of 10 µl. The primer pairs used to quantify different gene expression levels are shown in Supplementary Table 3. Fluorescence was measured at the end of each amplification step, and continuously during the melting curve analysis. Experiments were made in triplicate.

**Chemical analysis of glucose and metabolites** Glucose and lactate were determined by high-performance liquid chromatography (HPLC, sensitivity 50 mg/l). The compounds were separated using an Aminex HPX-87H, 300x7.8 mm column (Bio-Rad). The column temperature was maintained at 35°C and the flow rate at 0.4 ml/min. Organic acids (acetate and butyrate) were determined by gas chromatography (GC-3900, Varian) equipped with a flame ionization detector (sensitivity 20 mg/l) as described by Quéméneur *et al.*<sup>53</sup>

**Analysis of biogas production** Hydrogen (H<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) were quantified in the headspace by GC (Agilent 4890D) equipped with thermal conductivity detector (TCD). The gases were separated using a column GS-CARBONPLOT (30 m x 0.535 mm x 3.00 µm), the injector temperature was 80°C, the oven temperature was 40°C and the detector temperature was 90°C. The carrier gas was N<sub>2</sub> at high purity.

**Labeling with green or red fluorescent protein** The *gfp* and *mCherry* ORFs were amplified using the GAAACCGGTAAAACAAAGGAGGACGTTTATGGTGAGCAAGGGCGAGG GFP-Fwd/GATCGATGGTACCTTACTTGTACAGCTCGTCC GFP-Rev, and GAAACCGGTAAAACAAAGGAGGACGTTTATGGTGAGCAAGGGCGAGGAG mCherry-pB-Fwd/GATCGATGGTACCTTACTTGTACAGCTCGTCCATGCC mCherry – pB - Rev primers, respectively. The PCR amplification of *gfp* and *mCherry* was digested and introduced into the AgeI and KpnI sites of pBGF4 plasmid under the control of the hydrogenase constitutive

strong promoter as already described<sup>54</sup> to obtain pRD2 and pRD3 plasmid containing *gfp* and *mCherry*, respectively. Both are non stable plasmids in *C. acetobutylicum*. These different plasmids allowed the high expression of *gfp* and *mCherry* gene. The DNA sequences were analyzed by DNA sequencing (Cogenics, France). Next, pRD2 or pRD3 plasmid was electroporated into the *D. vulgaris* cells or transformed in *E. coli* DH10B. Electroporations were carried out as described by Bender and al<sup>54</sup> 2007 with minor modification. *D. vulgaris* cells were grown in Starkey medium to an optimal absorbance of 0.6 at 600 nm. Cells were harvested by centrifugation and washed with 50 ml of chilled, sterile electroporation wash buffer (30 mM Tris-HCl buffer at pH 7.2, not anaerobic). The resulting pellet was resuspended in 0.5 ml of chilled wash buffer. To 75 µl of cells 5 µl (4 µg) of plasmid was added and gently mixed. The mixture was transferred to a 2 mm gap electroporation cuvette (Molecular Bioproducts, San Diego, CA) and introduced to Gene Pulser MXcell system (Bio-Rad, France). The parameters for the electroporation were 1.75 kV, 25 µF and 250 Ω. The electroporated cells were diluted in 1 ml of anaerobic Starkey medium and incubated overnight at 37°C. 100-200 µl of overnight culture were plated in solid Starkey medium containing gentamycin. The plates were incubated anaerobically for 3 to 5 days at 37°C until gentamycin-resistant transformant colonies were visible.

**Labeling with Calcein-acetoxymethyl-ester (AM)** Calcein-AM is a small nonfluorescent derivative of calcein which is useful for differentiating between live and dead cells as is sufficiently hydrophobic to pass readily through cell membranes. Once it is inside, esterases cleave the AM groups, yielding the more hydrophilic calcein (623 Da), which is unable to cross membranes and is sequestered in the cytoplasm<sup>55</sup>. The loss of the acetomethoxy group also enables calcein to readily bind intracellular calcium, resulting in a strong yellowish-green fluorescence.

*D. vulgaris* cells were grown in Starkey medium<sup>10</sup> under anaerobic conditions and exponentially growing *D. vulgaris* (5 ml) was harvested at room temperature by centrifugation at 4000g for 10 min, washed twice with Starkey medium and resuspended in 5 ml fresh Starkey medium. 100 µl of calcein-AM (1 mg/ml in dimethylsulfoxide) were then added to the medium. The suspension was incubated in the dark at 37°C for 2 hours under anaerobic conditions. Cells were subsequently harvested and washed three times in fresh, dye-free GY medium and use in the exchange experiments.

The same protocol used for labeling *D. vulgaris* was followed to label *C. acetobutylicum* except that *C. acetobutylicum* cells were grown in 2 x YTG medium instead of Starkey.

**Study of exchange of cytoplasmic molecules** To study the molecules exchange between the two bacteria, *D. vulgaris* labeled were mixed with *C. acetobutylicum* unlabeled or *D. vulgaris* unlabeled were mixed with *C. acetobutylicum* labeled in 1:1 ratio according to the absorbance at 600 nm. In some cases, *C. acetobutylicum* labeled with calcein were mixed with *D. vulgaris* or *E. coli* labeled with mCherry. In some other cases, *D. vulgaris* labeled with calcein were mixed with *E. coli* labeled with mCherry. The mixture was diluted in 5 ml fresh GY medium and put in a tube containing a coverslip and incubated at 37°C for 24 h. The coverslip was removed after 24 h of growth and bacterial cells attached to coverslip were visualized by fluorescence confocal microscopy.

**Epifluorescence microscopy** Labeling of plasmic membrane and peptidoglycan of *C. acetobutylicum* and *D. vulgaris*. To investigate a possible point of contact between *D. vulgaris* and *C. acetobutylicum* in the co-culture, the cytoplasmic membrane was marked with FM4-64 fluorochrome (*N*-(3- triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide) and the peptidoglycan with WGA-FITC fluorochrome (wheat-germ agglutinin). The slides were observed using an automated inverted epifluorescence microscope



TE2000-E-PFS (Nikon) and the images were captured with a camera CoolSNAP HQ 2 (Roper Scientific) and analyzed with ImageJ52.

**Confocal microscopy** Determination of the transfer of calcein molecules, GFP and mCherry between *C. acetobutylicum* and *D. vulgaris* cells was carried out as follows: images of mixtures of labeled and unlabeled cells were acquired on a laser scanning confocal microscope FV 1000 (Olympus, Japan) using an Olympus UPLSAPO 100X (NA: 1.40) oil immersion objective. The excitation was provided by a multi-line argon-ion laser. Calcein and GFP were excited at 488 nm and mCherry at 543 nm. The microscope was controlled using Olympus Flu view 2.0c software

**Scanning Electron Microscopy (SEM)** was used to visualize in more detail a possible physical contact between *D. vulgaris* and *C. acetobutylicum* in the co-culture. The coverslips were immersed in the GY medium before sterilization. During the growth the coverslips were removed at different times of growth and treated as described by Dubey and Ben-Yehuda<sup>23</sup>, except for the last step of treatment, when they were dried chemically with hexamethyldisilazane (HMDS) for 30 minutes. Samples were then sputter-coated with gold and palladium (10 nm) with a FINE COAT-ion sputter JFC 1100 and examined using a field-emission gun scanning electron microscope (JEOL FEGSEM 6320F). Digital images were acquired with the NEWTEC System.

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**Author contributions:** SB, DR, ET and MTGO designed the research; SB, DR, AD, performed research; SB, DR, MLC, EL, YR, JH, ET, JPS, and MTGO analyzed the data; SB, DR, MLC, EL, ET and MTGO wrote the paper. SB and DR contributed equally to this work.

The authors declare no competing interests.

## Titles and legends to figures

**Figure 1. Growth of *D. vulgaris* and *C. acetobutylicum* in co-culture on GY medium. (a)** Absorbance curves for *D. vulgaris* in pure culture, *C. acetobutylicum* in pure culture, and co-culture, as indicated. **(b)** Glucose consumption in the two pure cultures and the co-culture. **(c,d)** numbers of genome copies in pure culture (c) and co-culture (d) as a function of time. The numbers of genome copies of each strain were determined by q-PCR, using the *dsrA* gene for *D. vulgaris* and the *endoG* gene for *C. acetobutylicum*. Notice that the pure culture of *D. vulgaris* does not consume glucose and does not grow. The right-hand panels show experiments in which open symbols represent bacteria shielded from one another by a dialysis membrane, and filled symbols represent results in pure cultures with no shielding. **(e)** Absorbance curves for *D. vulgaris* shielded from *C. acetobutylicum* and for *C. acetobutylicum* shielded from *D. vulgaris*. Controls of pure cultures are also shown. **(f)** Glucose consumption of *C. acetobutylicum* shielded from *D. vulgaris*, with a control of *C. acetobutylicum* in pure culture. **(g)** Numbers of genome copies determined as in (c). **(h, i)** Illustration of the experimental set-ups: **(h)** for the curves

related to shielded *D. vulgaris* and (i) for the ones related to shielded *C. acetobutylicum*. Results are means  $\pm$  s.d. (n=3).

**Figure 2. Scanning electron microscopy of *D. vulgaris* and *C. acetobutylicum* in pure culture and co-culture.** Exponentially growing *C. acetobutylicum* in 2YTG medium or *D. vulgaris* in Starkey medium under anaerobic conditions was diluted in fresh GY medium (for *C. acetobutylicum*) or fresh Starkey medium (for *D. vulgaris*), and transferred to Hungate tubes containing coverslips. For the co-culture, GY medium was used, and the bacteria were washed in GY medium before the co-culture. In all three cases the coverslips were removed after 30h growth, fixed with glutaraldehyde, dried chemically with hexamethyldisilane and coated with gold-palladium particles with a FINE COAT-ion sputter JFC 1100. Digital images were acquired with the NEWTEC system. (a, b) Images of *D. vulgaris* in the Starkey medium (a) and *C. acetobutylicum* in GY medium (b) in pure cultures. (c) Images of the two bacteria in co-culture. (d) Image of a *D. vulgaris* cell in close contact with a *C. acetobutylicum* cell. Images of the boxed regions are shown at higher magnification at the right. Scale bar = 1  $\mu$ m in all panels.

**Figure 3. Exchange of calcein between *D. vulgaris* and *C. acetobutylicum*.** *D. vulgaris* growing exponentially in Starkey medium was labeled with calcein, washed with GY medium, mixed with unlabeled *C. acetobutylicum*, and visualized by fluorescence confocal microscopy at time zero (a), or after 24 h incubation at 37°C in GY medium (b). (c) A bigger field. (d,e) Exponentially growing *C. acetobutylicum* labeled with calcein was washed, mixed with unlabeled *D. vulgaris* and visualized at time zero (d) or after 24 h incubation at 37°C in GY medium (e). Non-fluorescent cells are in yellow or white boxes, as labeled. Scale bar = 2  $\mu$ m.

**Figure 4. Bidirectional transfer of calcein and mCherry between *C. acetobutylicum* and *D. vulgaris*.** (a) *D. vulgaris* growing exponentially in Starkey medium was labeled with mCherry (red), washed in GY medium, mixed with *C. acetobutylicum* labeled with calcein (green), and visualized by fluorescence confocal microscopy at time zero or after 24 h incubation at 37°C in



GY medium. Notice that at time zero, each type of bacteria shows only green or red fluorescence, but after 24h *C. acetobutylicum* originally green, shows red fluorescence, and *D. vulgaris* originally red, shows green fluorescence. **(b)** Results from an equivalent experiment showing exchange of fluorescent labels in both directions (yellow). White arrows indicate apparent connections between bacterial cells. Scale bar = 2  $\mu\text{m}$  in all panels.

**Figure 5. Effect of various conditions on transfer of calcein from labeled *C. acetobutylicum* to *D. vulgaris*.** All results are shown after 24 h of co-culture, and in all panels the scale bars = 2  $\mu\text{m}$ . **(a)** No transfer when *C. acetobutylicum* is shielded from *D. vulgaris* by a dialysis membrane and cultivated in GY medium at 37°C (*D. vulgaris* shows no fluorescence) and **(b)** the *C. acetobutylicum* remains labeled. **(c)** Transfer of calcein from *C. acetobutylicum* to *D. vulgaris* in co-culture in GY medium supplemented with 20 mM lactate (without shielding). **(d)** No transfer observed in GY medium supplemented with 20 mM sulfate in addition to 20 mM lactate. A great number of *D. vulgaris* cells (which can grow on sulfate) can be seen, but they show no green fluorescence, indicating that the transfer has been greatly reduced (or is non-existent). **(e)** A scanning electron micrograph made in the same conditions as (d). Scale bar = 2  $\mu\text{m}$  in all panels.

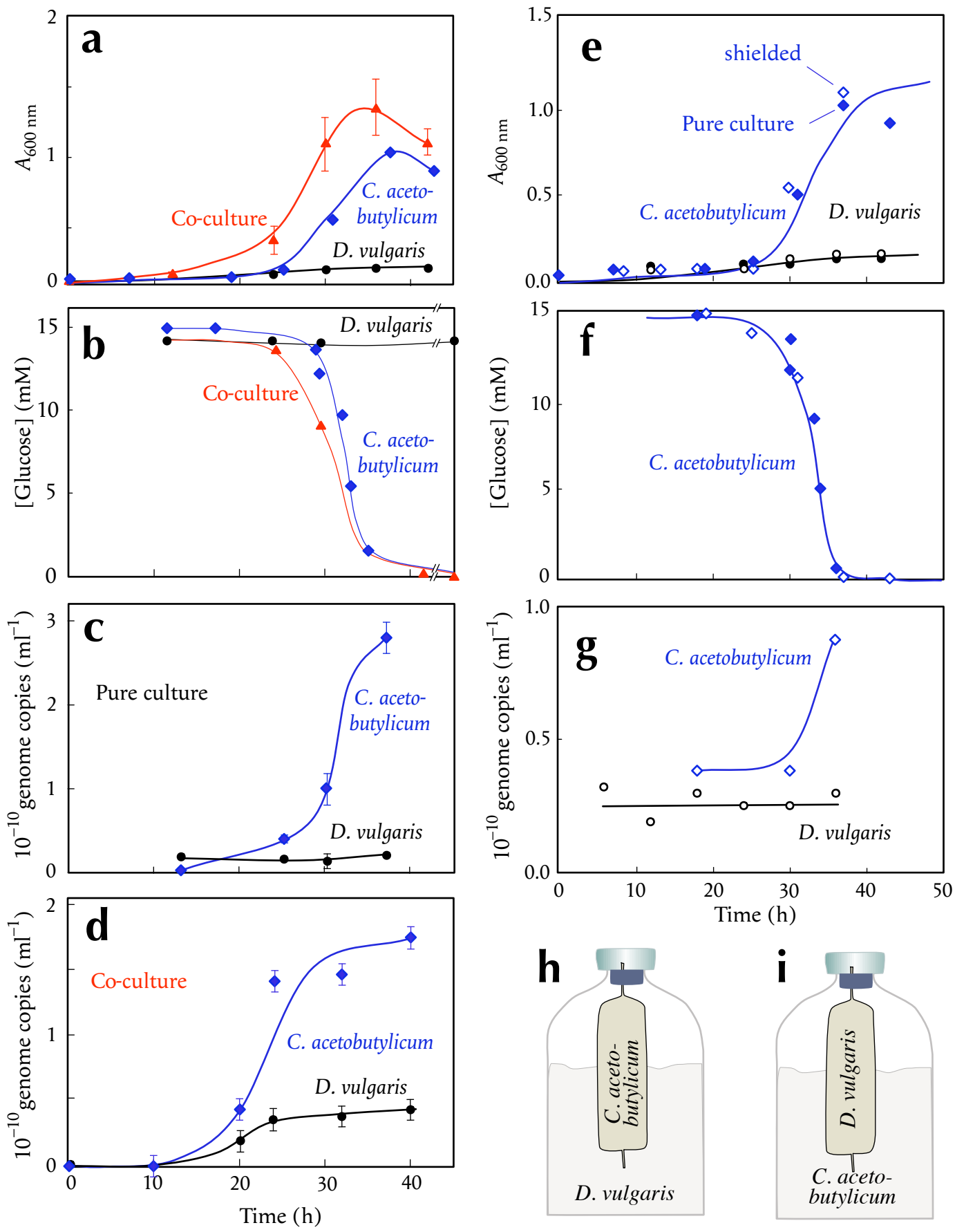
**Figure 6. Absence of exchange of fluorescent molecules between *E. coli* and *C. acetobutylicum*.** **(a)** Exponentially growing *C. acetobutylicum* labeled with calcein (green) was mixed with *E. coli* labeled with mCherry (red), and the mixture was visualized by fluorescence confocal microscopy at time zero or after 24h of incubation at 37°C. Notice that in contrast to Figure 4 no yellow color can be seen in the overlay, and no *C. acetobutylicum* shows red fluorescence. Scale bar = 2  $\mu\text{m}$  in all panels.

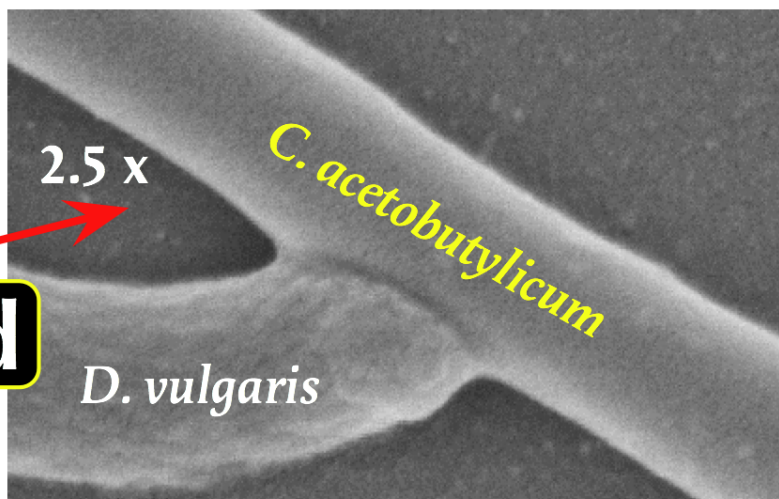
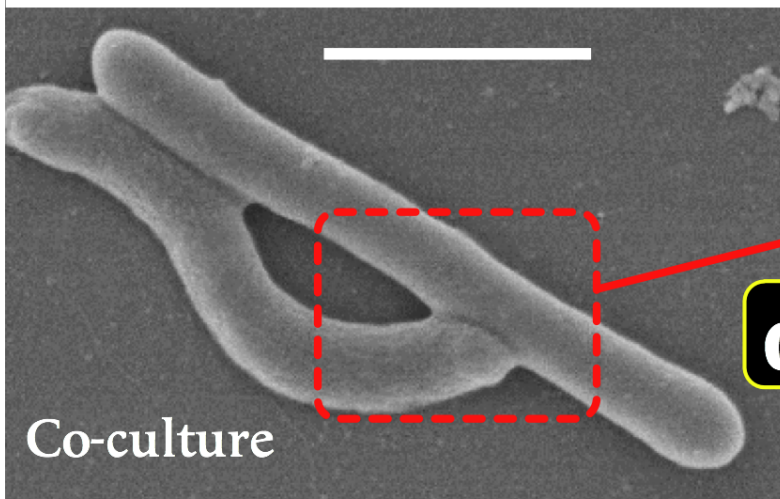
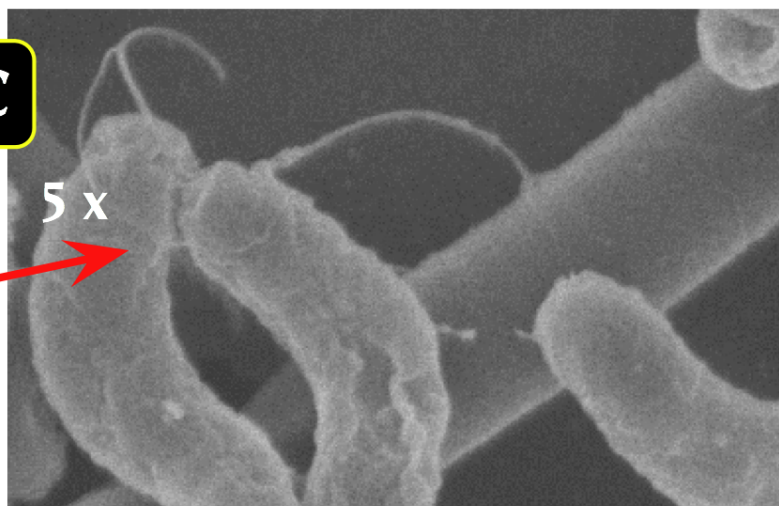
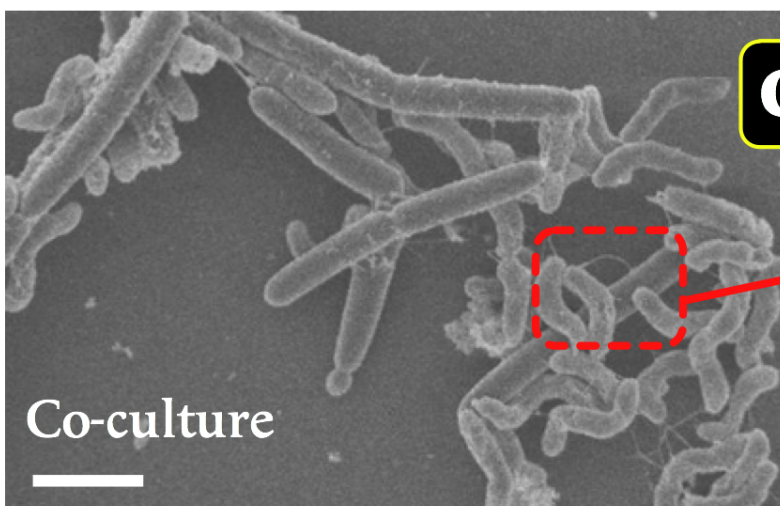
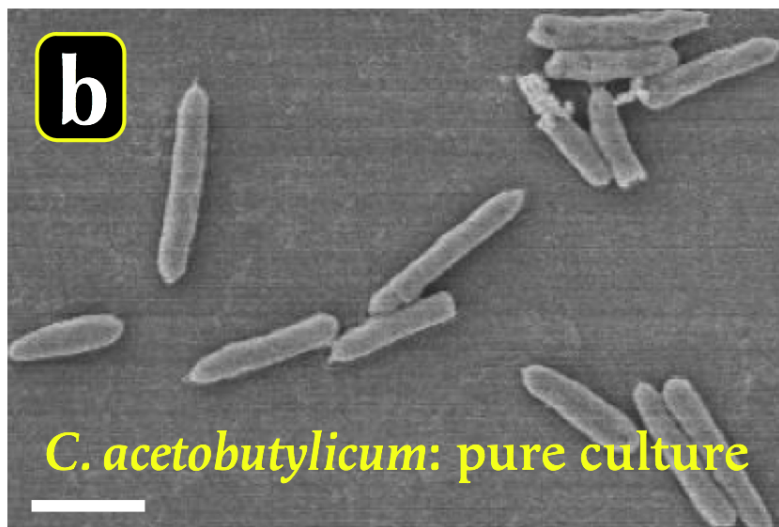
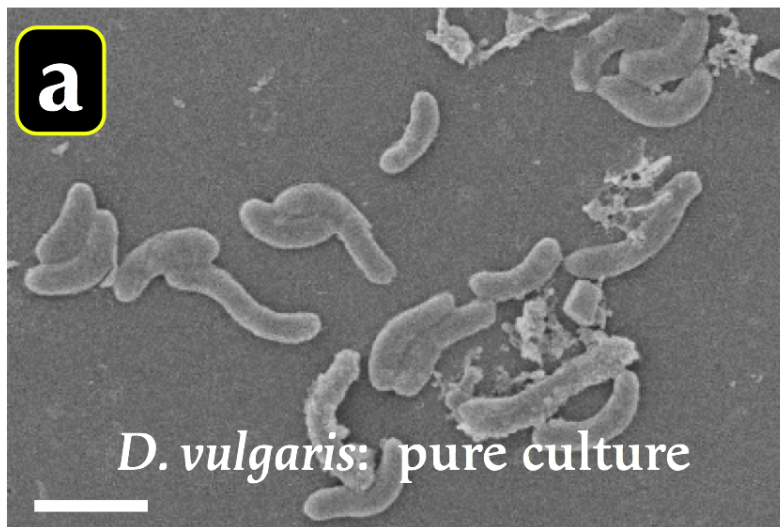
**Figure 7. Exchange of fluorescent molecules between *E. coli* and *D. vulgaris*.** **(a)** Exponentially growing *D. vulgaris* labeled with calcein (green) was washed with GY medium, mixed with *E. coli* labeled with mCherry (red), and the mixture was visualized at time zero or after 24 h incubation at 37°C. Yellow color, indicating exchange of both labels, is visible in the

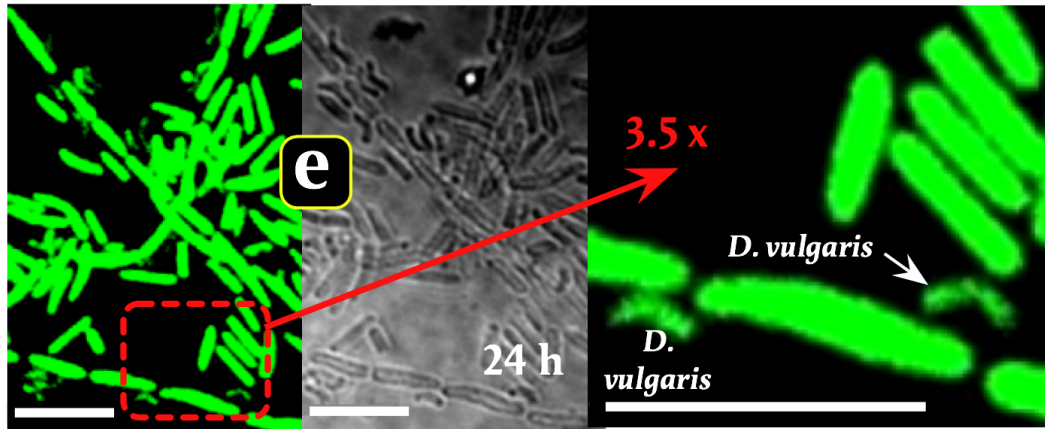
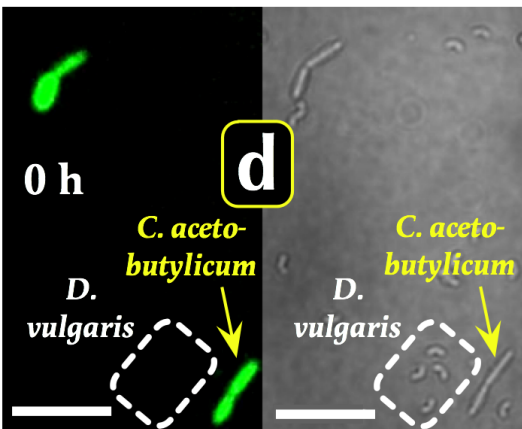
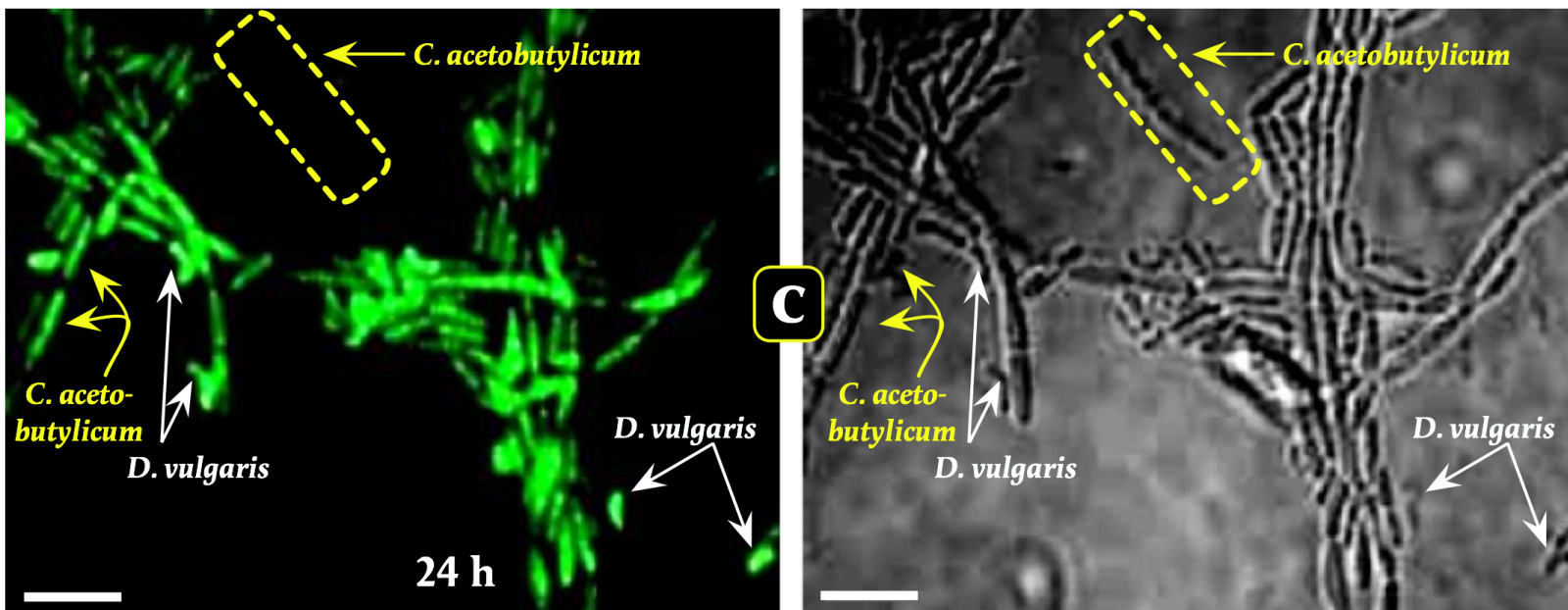
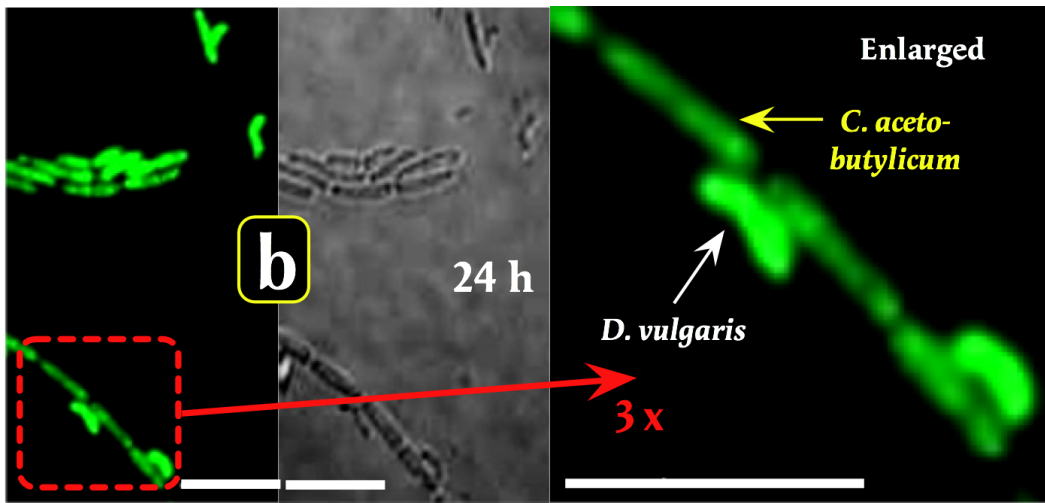
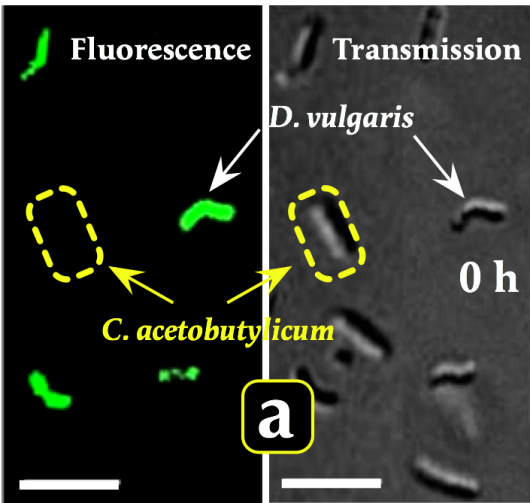
overlay. **(b)** A similar experiment with a bigger field. Notice the many cells with both labels (yellow). The white arrows indicate connections between *E. coli* and *D. vulgaris*. Scale bar = 2  $\mu\text{m}$  in all panels.

**Figure 8. Time courses of glucose consumption and metabolite production of pure culture of *C. acetobutylicum* and co-culture.** **(a)** Consumption of glucose, and production of butyrate, acetate and lactate in pure culture. **(b)** Production of  $\text{H}_2$  and  $\text{CO}_2$ . **(c)** Consumption of glucose and production of anions in co-culture. **(d)** Production of gases in co-culture. **(e)** Schematic representation of the Hungate tube used for the culture. **(f)** Production of  $\text{H}_2$  by *C. acetobutylicum* in pure culture, in co-culture and shielded (with *D. vulgaris* in a dialysis tube, as in Fig. 1i), as labeled. Results are means  $\pm$  s.d. (n=3).

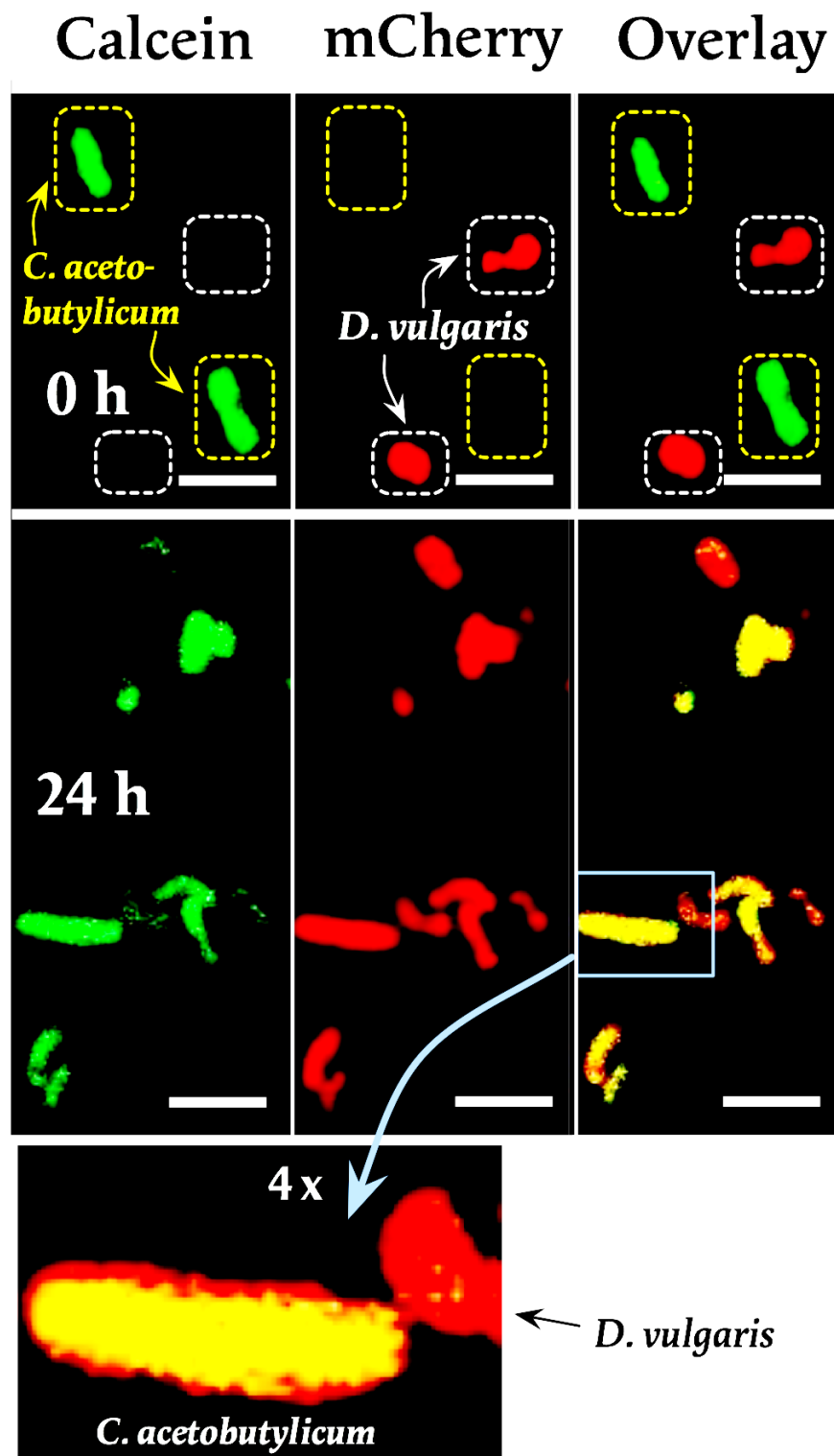




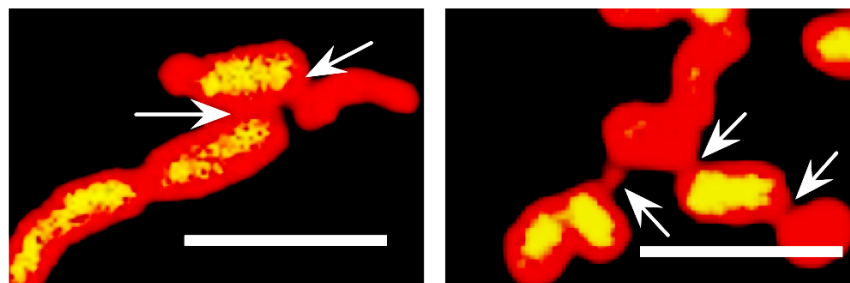




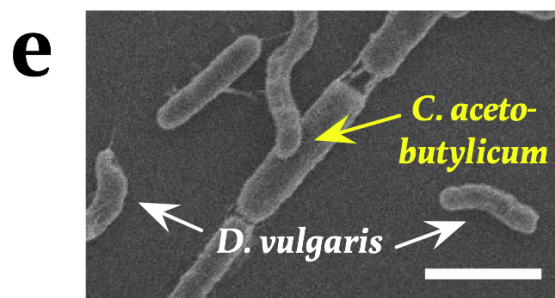
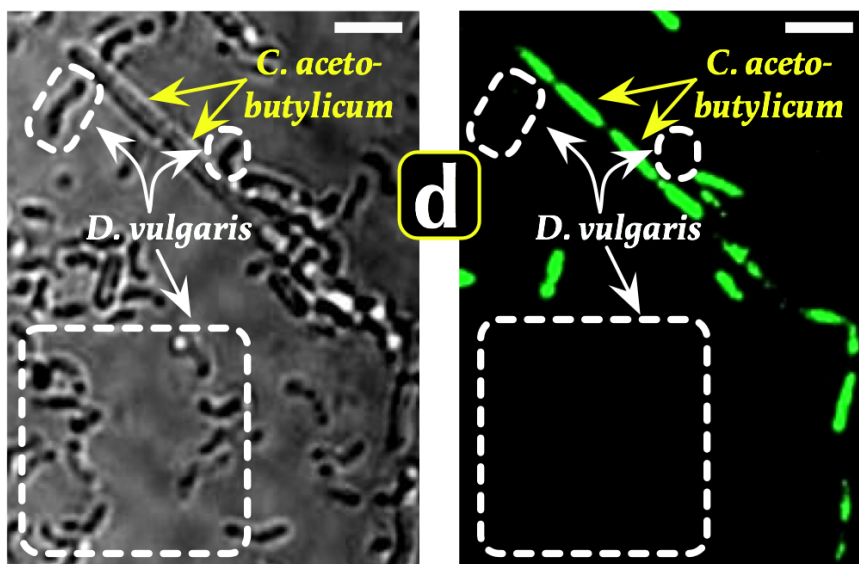
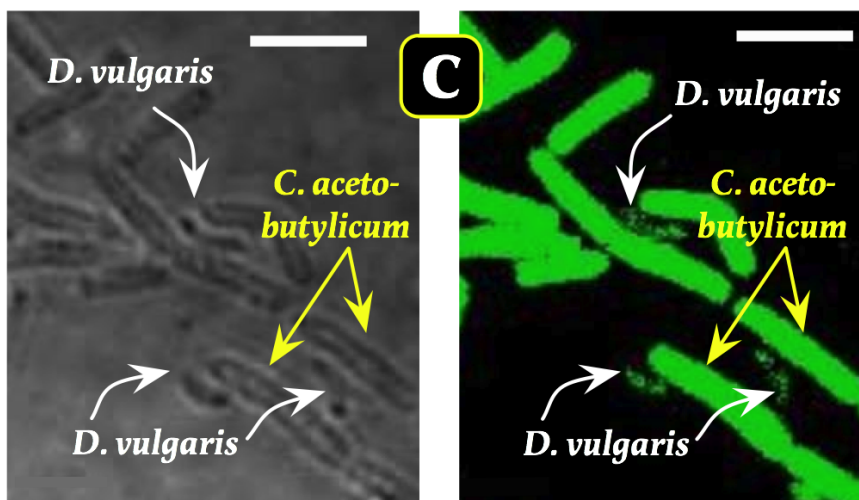
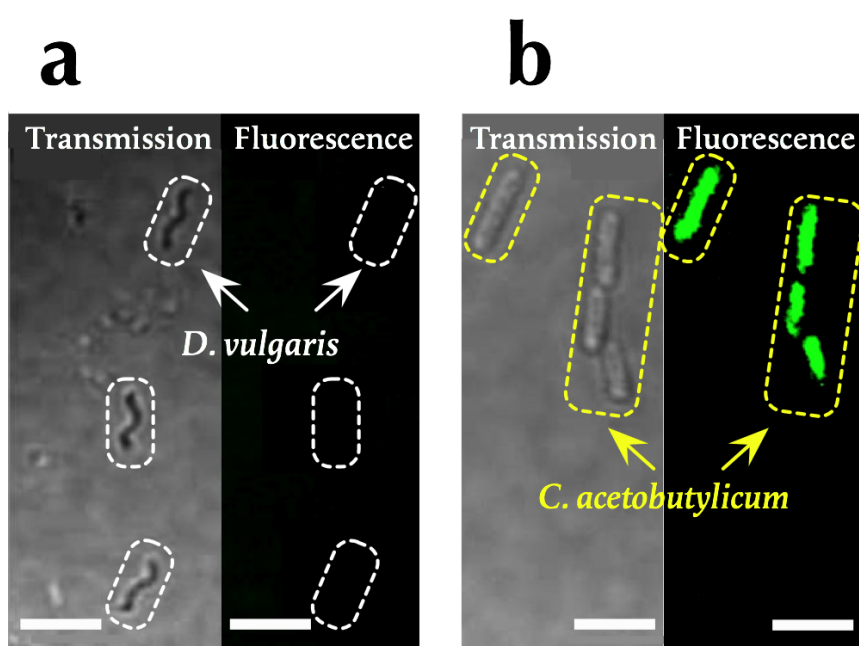
**a**



**b**







mCherry

*E. coli*

0 h



Calcein

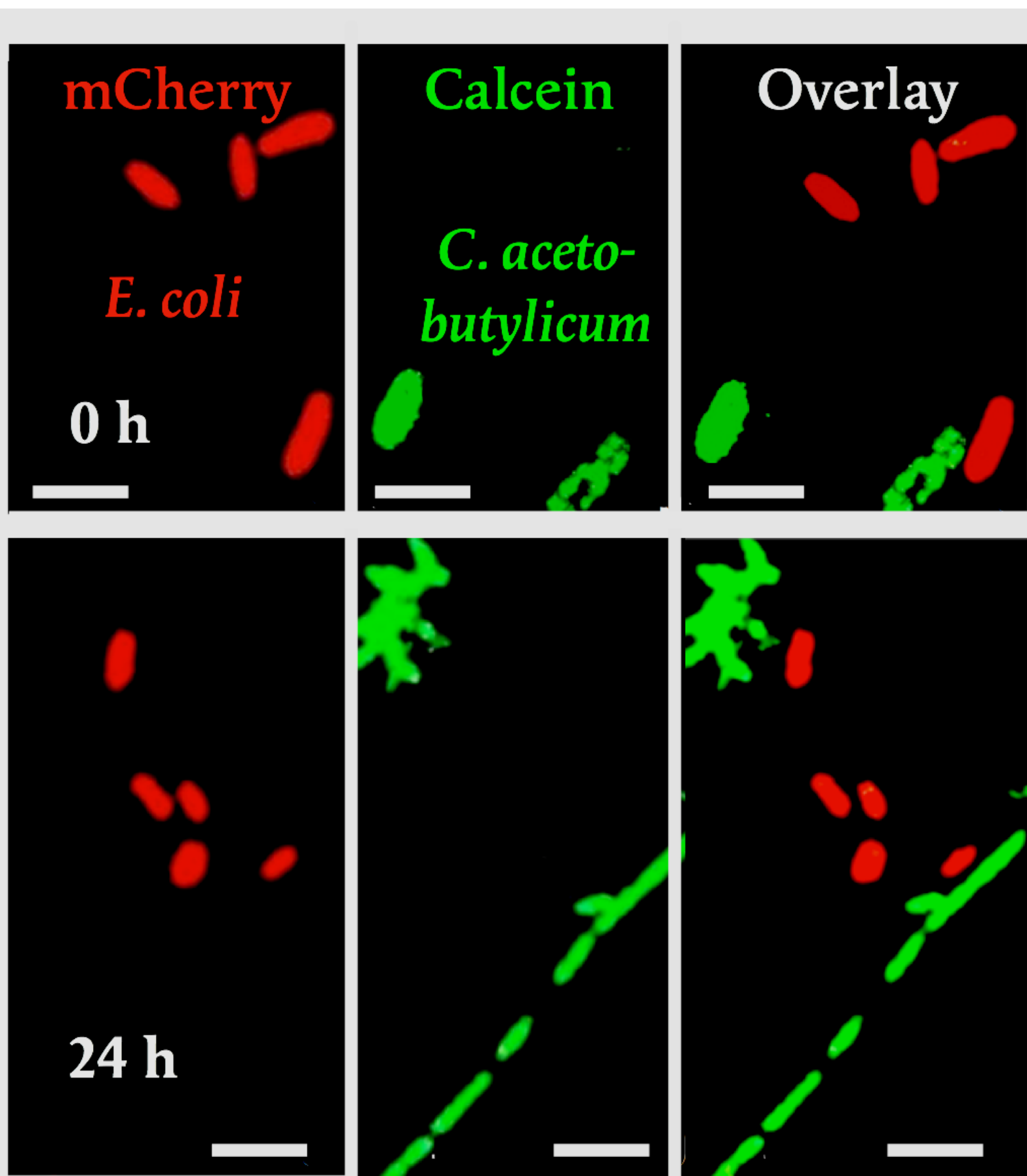
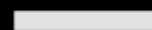
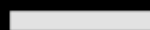
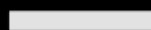
*C. acetobutylicum*

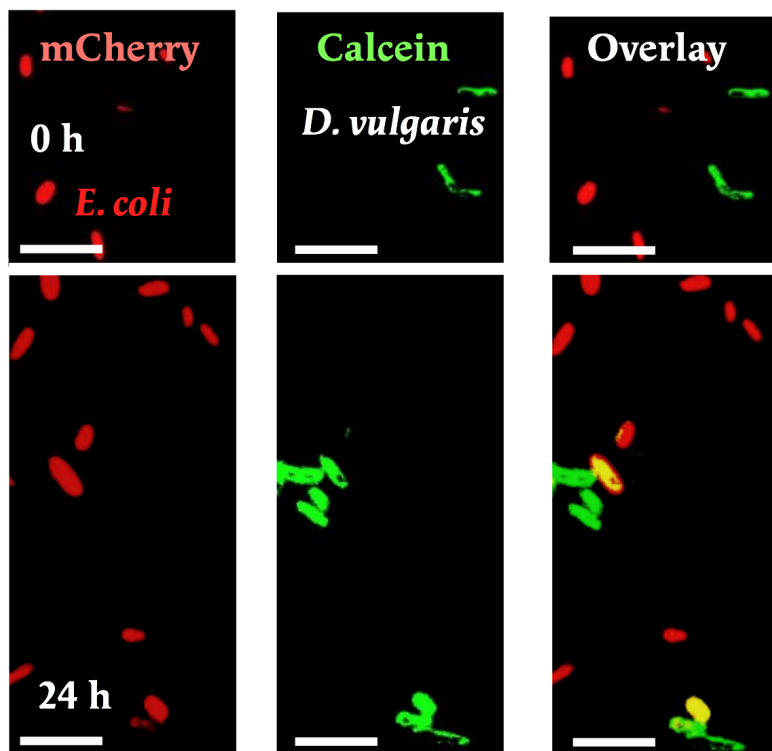


Overlay



24 h



**a****b**